



18

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Re application of: Glazer et al.

Group Art Unit: Not yet assigned

Serial No. 10/617,208

Examiner: Not yet assigned

Filed: July 10, 2003

Attorney Docket No. B00-016-2

For: *Multifunctional Recombinant  
Phycobiliprotein-Based Fluorescent  
Constructs and Phycobilisome  
Display*

DECLARATION UNDER 37CFR1.131

1. We are the coinventors of the subject patent application.
2. Attached Exhibit IV is photocopies of 15 pages from a laboratory notebook maintained by inventor Yuping Cai during his tenure in the laboratory of inventor Alexander Glazer, and describing our work performed between November 1997 and April 1998 in the United States and which demonstrates our production of (i) a fusion protein comprising a functional displayed domain and a functional phycobiliprotein domain incorporated in a functional oligomeric phycobiliprotein; (ii) a cell comprising a functional oligomeric phycobiliprotein comprising a fusion protein comprising a functional displayed domain and a functional phycobiliprotein domain; and (iii) a fusion protein comprising a functional displayed domain and a functional phycobiliprotein domain incorporated in a functional oligomeric phycobiliprotein, wherein the oligomeric phycobiliprotein provides a fluorescent tag.
3. In particular, the 15 pages are from notebook "YAC #10" section "GCN4pLI Tetramers", and describe experimental result showing the use of Strep-tagged phycocyanin to fluorescently stain streptavidin-coated agarose beads:

Pages 1 to 7: making of expression plasmid pBS323 encoding the HisTag-StrepII-pLI-CpcA fusion protein; and plasmid map and relevant information.

Page 8: purification of the fusion protein from E. coli cells.

Pages 9-15: purification from Anabaena cells, and characterization of An323 (HisTag-StrepII-pLI-CpcA:CpcB recombinant protein); and use of An323 to stain streptavidin-coated agarose beads (page 10). The binding of the StrepII tag-containing recombinant phycobiliprotein An323 to streptavidin was shown to be specific.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

A. N. Glazer

Alexander N. Glazer

08/02/03

Date

Yuping Cai

Date

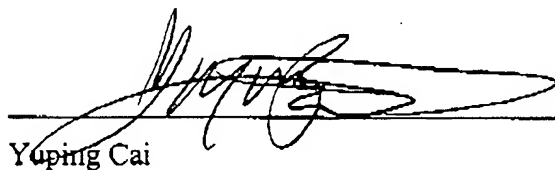
Pages 9-15: purification from Anabaena cells, and characterization of An323 (HisTag-StrepII-pLI-CpcA:CpcB recombinant protein); and use of An323 to stain streptavidin-coated agarose beads (page 10). The binding of the StrepII tag-containing recombinant phycobiliprotein An323 to streptavidin was shown to be specific.

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Alexander N. Glazer

Date



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Yuping Cai

Date

9/2/03

To make Histag-StrepTag2-GCN4pLI-CpCA:

$\left\{ \begin{array}{l} \text{pBS309} \times \text{NdeI} + \text{HindIII} \rightarrow 4.7 \\ \text{pANcpcA} \times \text{NdeI} + \text{HindIII} \rightarrow 0.45 \end{array} \right.$

11/21/97. Digestions done by Hardy. Look like there is a extra NdeI site in pBS309? Need to recheck.

12/10/97. The pBS309 is remade and cleared up.



12/18/97. Digestions done by Hardy. Bands cut out, combined, and stored at  $-20^{\circ}\text{C}$ .

12/19/97. Genecleaned and ligated.

12/20/97. Ligation mix used to transform DH5 $\alpha$ . Selection: LA + Sp100.  $30^{\circ}\text{C}$ .

12/22/97. About 300 transformants on the  $1/10$  plate. More on the  $9/10$  plate. Eight picked for miniprep.

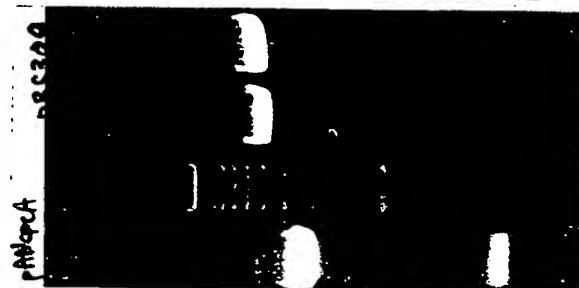
12/24/97. Minipreps made by Yuning.

12/26/97. Digestion of the eight minipreps w/ NdeI + HindIII shows that none has the 0.45-kb insert. Need to repeat.

12/29/97. Digestions repeated. Bands cut out, combined, and stored at  $-20^{\circ}\text{C}$ .

1/7/98. Genecleaned and ligated.

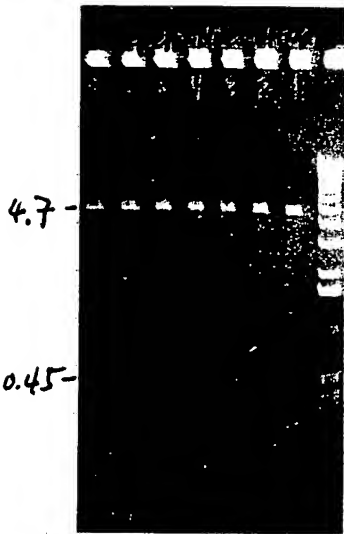
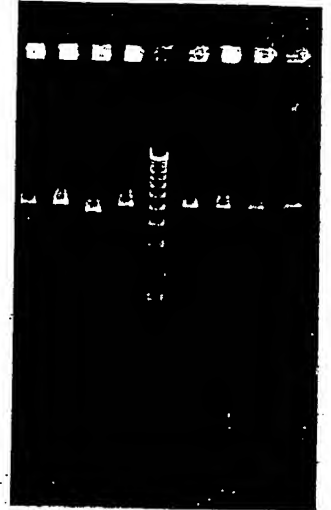
1/8/98. Ligation mix used to transform DH5 $\alpha$ . Selection: LA + Sp.  $30^{\circ}\text{C}$ . Put to R/T the next day.



1/12/98. About 200 transformants on the 1/10 plate, more on the 9/10 plate. Eight picked for minipreps.

1/13/98. Minipreps made by Hardy.

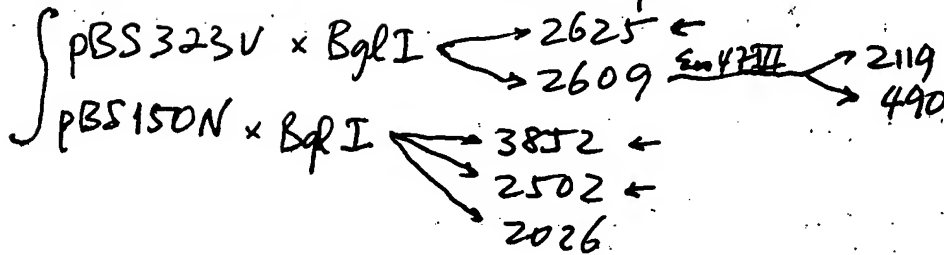
1/14/98. Digestion of the eight minipreps (by Steve) with Bsp<sup>1</sup>MI (accidental) shows that #6 doesn't have an insert. The rest need to be checked with NdeI + HindIII.



1/15/98. Digestion of the select minipreps (by Hardy) with NdeI + HindIII shows that every one is right.

#4 is saved as pBS323V (5,234 bp)  
#4 and #5 used to isolate Ec323.  
Both cultures give high yield of the expected 25.5 kD protein. (see gel).

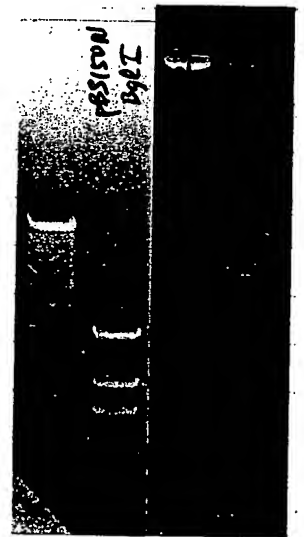
To make pBS323:



1/16/98. Digestions done. Bands cut out, combined.

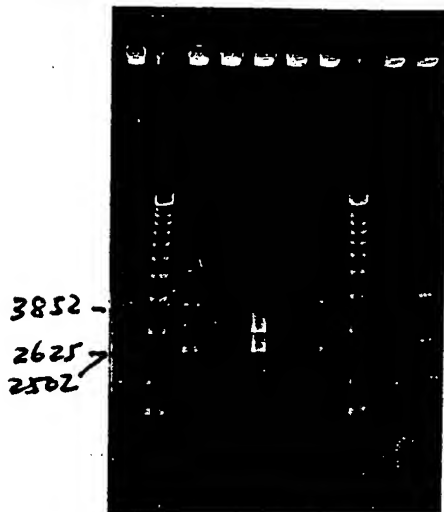
1/21/98. Gene cleaned and ligated.

1/22/98. Ligation mix used to transform DH5 $\alpha$ .  
Selection: LA + Sp100, 30°C.



1/24/98. About 200 transformants on the 1/10 plate. More on the 9/10 plate. Eight picked for minipreps.

1/27/98. Minipreps made by Wendy.



1/28/98. Digestion of the eight min:preps (by Steve) with BglI shows that all but #5 look correct.  
#7 saved as pBS323 (8,979 bp).

1/29/98. Plasmid DNA of pBS323 used to transform HB101 (pRI528).  
Selection: LA + Cm + Sp. 30°C.

1/31/98. Hundreds of transformants obtained.

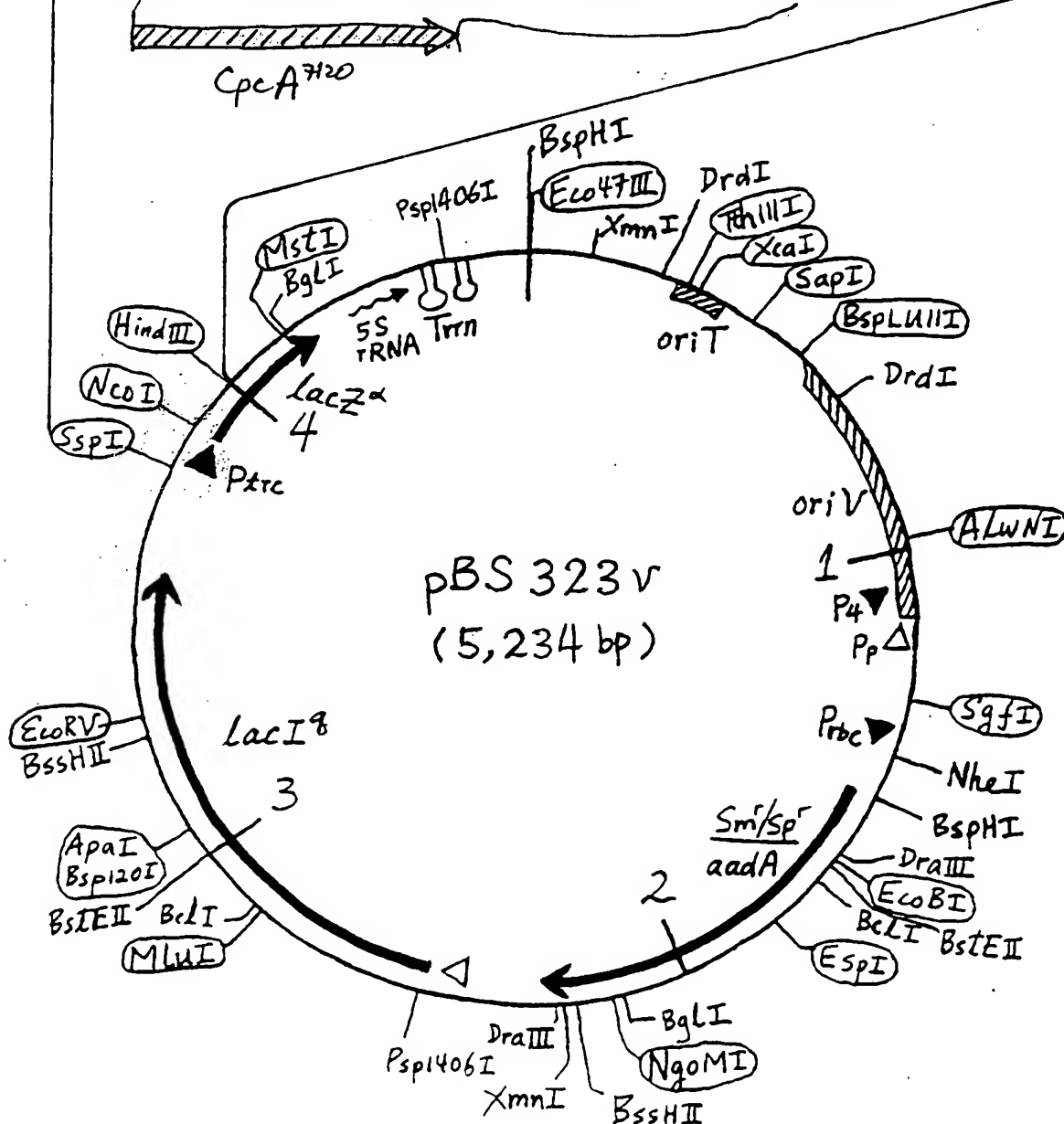
2/1/98. Mating w/ WT7120 performed.

2/14/98. All cell spots have grown some. The mating filter is transferred to AA@Sm1.0Sp10 plate. Put back under HL.

2/19/98. The control spots are dying, and exconjugants are seen. The mating filter is transferred to a new plate of AA@Sm1.0Sp10. Put under HL.

2/26/98. Control spots are mostly dead, and lots of exconjugants are seen. Some used to streak on new plates of AA@Sm1.0Sp10, and inoculated AA/8@Sp10 flask cultures. Put under HL.

4/17/98. Flask cultures of *Anabaena PCC7120* (pBS323) look yellowish and reddish fluorescent. Streaks on plates also look unhealthy.



AATATTCTGAAATGAGCTGTGACAAATTAATCATCCGGTCCGTATAATCTGTGGAATTGTGA  
 SspI -35 -10 +1

binding site ---|<-----His tag----->|  
 Met Gly His His His His His His  
 GCGGATAACAATTTTCACACAGGAAACAGACC ATG GGT CAT CAT CAT CAT CAT CAC  
 RBS NcoI

<----- StrepTag-II ----->|  
 Ala Ser Asn Trp Ser His Pro Gln Phe Glu Lys Gly  
 GCT AGT AAC TGG TCA CAC CCA CAA TTC GAG AAA GGT  
 NheI BspMII

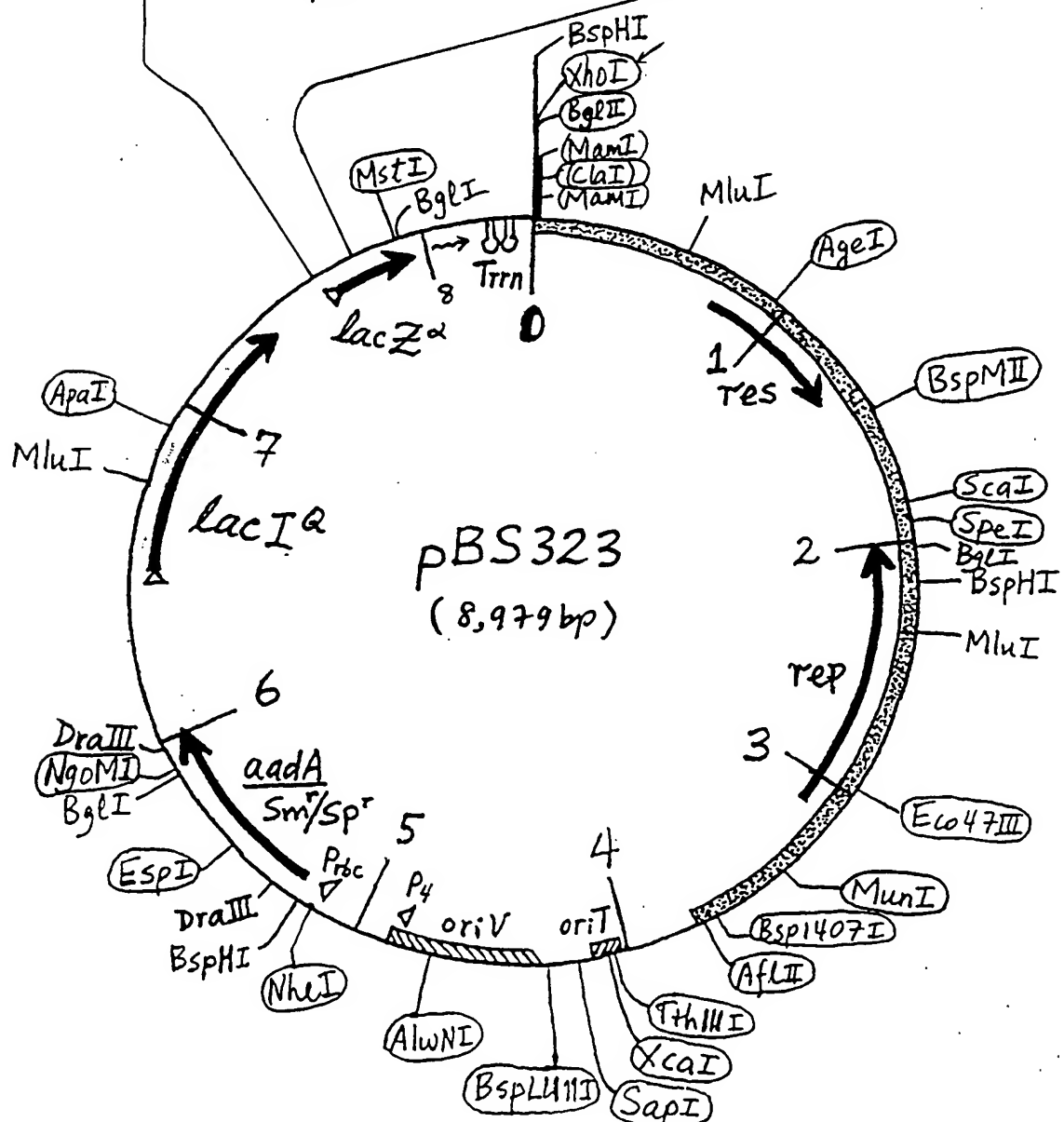
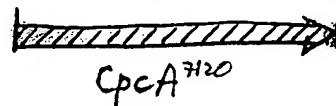
Exhibit IV  
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Ala Ser Ser Gly Arg Met Lys Gln Ile Glu Asp Lys Leu Glu Glu Ile  
 GCT AGC TCC GGA CGC ATG AAA CAA ATT GAA GAT AAG TTA GAG GAA ATT  
 NheI BspMII

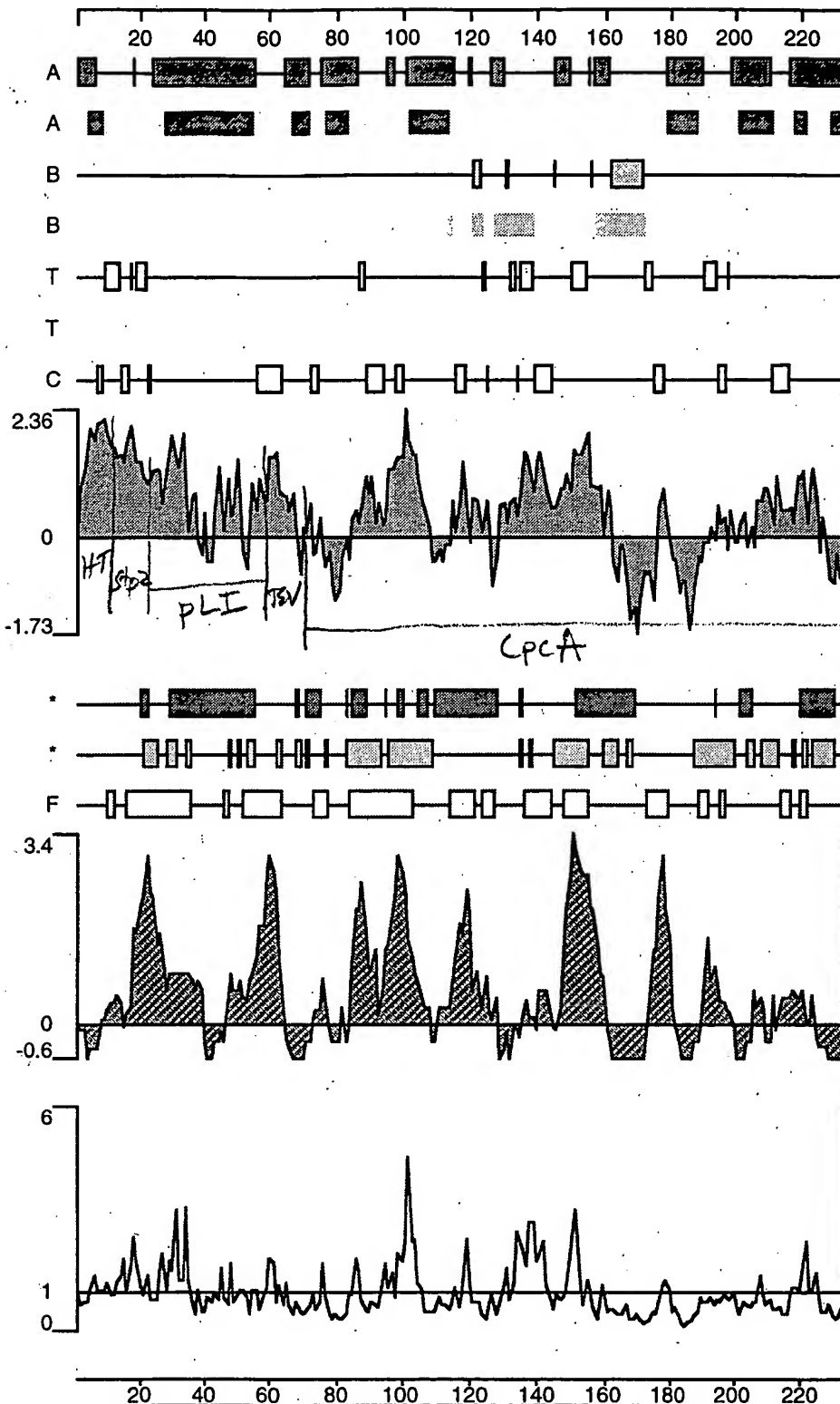
Leu Ser Lys Leu Tyr His Ile Glu Asn Glu Leu Ala Arg Ile Lys Lys  
 CTT TCG AAA CTC TAT CAC ATT GAA AAT GAG TTA GCC CGC ATT AAG AAA  
 AsuII

Leu Leu Gly Glu Arg Gly Thr Gly Glu Asn Leu Tyr Phe Gln Gly Ala  
 TTA CTC GGC GAA CGC GGT ACC GGT GAA AAC CTG TAT TTT CAG GGC GCC  
 KpnI AgeI EheI

His Met Gly Ile Gln Arg Pro Thr Ser Thr Arg Ala Ser Leu Ala Leu  
 CAT ATG GGA ATT CAA AGG CCT ACC TCG AGG AGG GCA AGC TTG GCA CTG  
 NdeI EcoRI StuI Sall HindIII







■ Alpha, Regions - Garnier-Robson

■ Beta, Regions - Garnier-Robson

□ Turn, Regions - Garnier-Robson

□ Coil, Regions - Garnier-Robson

■ Hydrophilicity Plot - Kyte-Doolittle

■ Alpha, Amphipathic Regions - Eisenberg

■ Beta, Amphipathic Regions - Eisenberg

□ Flexible Regions - Karplus-Schulz

■ Antigenic Index - Jameson-Wolf

□ Surface Probability Plot - Emini

Predicted Structural Class of the Whole Protein: Alpha  
Deléage & Roux Modification of Nishikawa & Ooi 1987

Exhibit IV  
Page 7 of 15

Analysis	Whole Protein
Molecular Weight	25525.70 m.w.
Length	233
1 microgram =	39.176 pMoles
Molar Extinction coefficient	25580±5%
1 A(280) =	1.00 mg/ml
Isoelectric Point	7.65
Charge at pH 7	1.90

Whole Protein Composition Analysis

Amino Acid(s)	Number count	% by weight	% by frequency
Charged (RKHYCDE)	72	38.96	30.90
Acidic (DE)	24	11.70	10.30
Basic (KR)	24	13.37	10.30
Polar (NCQSTY)	64	28.77	27.47
Hydrophobic (AILFWV)	78	30.74	33.48
A Ala	30	8.36	12.88
C Cys	1	0.40	0.43
D Asp	8	3.61	3.43
E Glu	16	8.09	6.87
F Phe	6	3.46	2.58
G Gly	21	4.70	9.01
H His	12	6.45	5.15
I Ile	14	6.21	6.01
K Lys	12	6.03	5.15
L Leu	21	9.31	9.01
M Met	3	1.54	1.29
N Asn	10	4.47	4.29
P Pro	7	2.66	3.00
Q Gln	11	5.52	4.72
R Arg	12	7.34	5.15
S Ser	17	5.80	7.30
T Thr	14	5.54	6.01
V Val	5	1.94	2.15
W Trp	2	1.46	0.86
Y Tyr	11	7.03	4.72
B Asx	0	0.00	0.00
Z Glx	0	0.00	0.00
X Xxx	0	0.00	0.00
. Ter	0	0.00	0.00

323.

25.5 KD

HisTag-Strp2 - GCN4pLI - CpcA

25,525.70

+ 585 (PCB)

26,110.70

fully chromophorylated

+ 19,572.00 (P)

45,682.70

EC323V-4  
5

4/20/98 by STEVE

**Isolation of 6xHis-tagged proteins in GuHCl denaturing condition**

(for checking proteins on SDS-PAGE) Cell pellets from ~~800~~ 1 liter cultures should be frozen in 400-ml centrifuge tubes at -20°C

Grown up at 30°C, O/N.

IPTG-induced at 37°C for 7 hrs

1. Add the following to solubilize cells:

30 ml **buffer G**25 ul  $\beta$ -mercaptoethanol (final ~ 10 mM)300 ul 100x **PMSF**1 ml 20% **Triton X-100** (final ~ 0.5%)

close bottle and shaker on 30°C shaker for 1 hr.

Exhibit IVPage 8 of 15

2. Transfer everything to a 40-ml Oakridge tube, balance well, then centrifuge at 15,000 rpm for 20 min at 4°C.

3. Transfer supernate to a new 50-ml tube, add 2 to 3 ml  $\text{Ni}^{2+}$ -NTA beads suspension, rock in coldroom for 20 min. for His-tagged proteins to bind.

4. Pour in 1.5-cm (diam.) column, let drain. Reload 2x;

5. Wash with  $\geq 10$ x bed volume **buffer G**;

6. Wash with  $\geq 10$ x bed volume **buffer GA**;

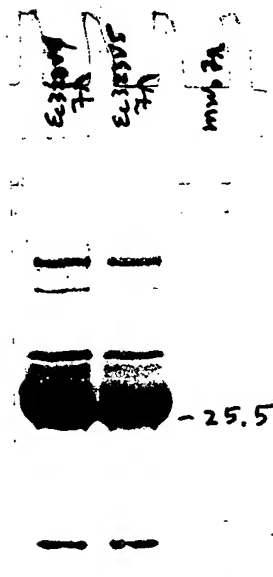
7. Elute with 3 ml **buffer GC**, 2x;

If proteins are to be renatured, after step 7 the eluate should be diluted with buffer GA, then dialyze against appropriate buffer (such as 20 mM Tris-HCl pH 8.0, 150 mM NaCl). If the isolated protein contains the core streptavidin (StvC) moiety, the protein needs to be rid of bound biotin by dialysis against 1 to 2 L of 6 M GuHCl pH 1.5 (Urea can not denature StvC completely).

8. Transfer the 6 ml eluate to a dialysis tubing, dialyze against 4 L of  $\text{H}_2\text{O}$  in coldroom for 2 hr to overnight.

9. If lots of proteins crash out of solution (as expected), retrieve dialysis mixture, spin, dissolve the precipitate with 1x SDS buffer. If not much precipitate, precipitate proteins in the supernate with equal volume of 20% **TCA**.

- 10 Run SDS-PAGE to check proteins.

Moderate amount of crash out.  
Very little proteins in the supernate (by spec.).

Very high yield of the expected 25.5 kD protein ;

5/20/98.

# An323 (1st)

3/14/98. A 2-L culture of *Anabaena PCC7120* (pBS323) inoculated from several flask cultures (washed 2x), into AAA(1/2 + 3 mM HEPES pH 7.0 + Sp20. Put under HL, bubbled.

3/19/98. The culture has grown to a moderately dense one. relatively healthy-looking. yellowish green. DFTC added to final 0.5 mM. Put back under HL. bubbled.

3/23/98. The culture has grown to a near black one. whole-cell spec: 80323402.sp. Cells harvested by centrifugation — supernate medium is light yellowish green. Wet cell weight = 1.6 + 1.7 + 2.2 gm. Stored at -20°C

3/27/98. The 2.2 gm cell pellet used to make An323 protein.  
(by man/Steve/Yuping, see attached sheets).

12.5 ml total after dialysis.

$$A_{622} = A_{623} = 0.1933 \times 10 = 1.933$$

$$\begin{aligned} [\text{mg/ml}] &= \frac{A}{\epsilon} \cdot \text{MW} \\ &= \frac{1.933}{290,000} \times 45,682.70 \\ &= \underline{0.3 \text{ mg/ml}} \end{aligned}$$

$$[M] = \frac{1.933}{290,000} = \underline{6.7 \mu\text{M}}$$

4/2/98. Use stvC-Agarose beads to test binding.

stvC-Agarose beads from Sigma:

50% suspension. Specification: 1 ml packed gel binds 23  $\mu$ g Biotin.  
MW Biotin = 244.31; 23  $\mu$ g  $\Rightarrow$  0.09414  $\mu$ Mole  
= 94.14 nMole

So: 2 ml bead suspension = 1 ml packed beads  
have 23.5 nMole immobilized stvC.

$\Rightarrow$  2.35 nMole stvC/100  $\mu$ l bead suspension.

$\approx$  1.2 nMole stvC/100  $\mu$ l bead suspension.

$\approx$  4.7 nMole binding site/100  $\mu$ l bead suspension.

An323: 6.7 nMole monomer/ml,

1.4 ml  $\Rightarrow$  9.4 nMole monomer.

$\Rightarrow$  2:1 binding to 100  $\mu$ l Bead suspension.

An321: 8.83 nMole monomer/ml.

1.064 ml  $\Rightarrow$  9.4 nMole monomer

$\Rightarrow$  2:1 binding to 100  $\mu$ l Beads suspension as control.

Beads washed w/ buffer  $\Phi$  2x.

look under UV: An323 beads

much more fluorescent than An321.

Before Biotin

To be sure of specific binding. Beads washed 2x w/ 100  $\mu$ M Biotin in

buffer  $\Phi$ . Then look at UV:

Most color on An323 beads gone?

after Biotin

$\Rightarrow$  Specific binding of An323 to stvC?



321

323

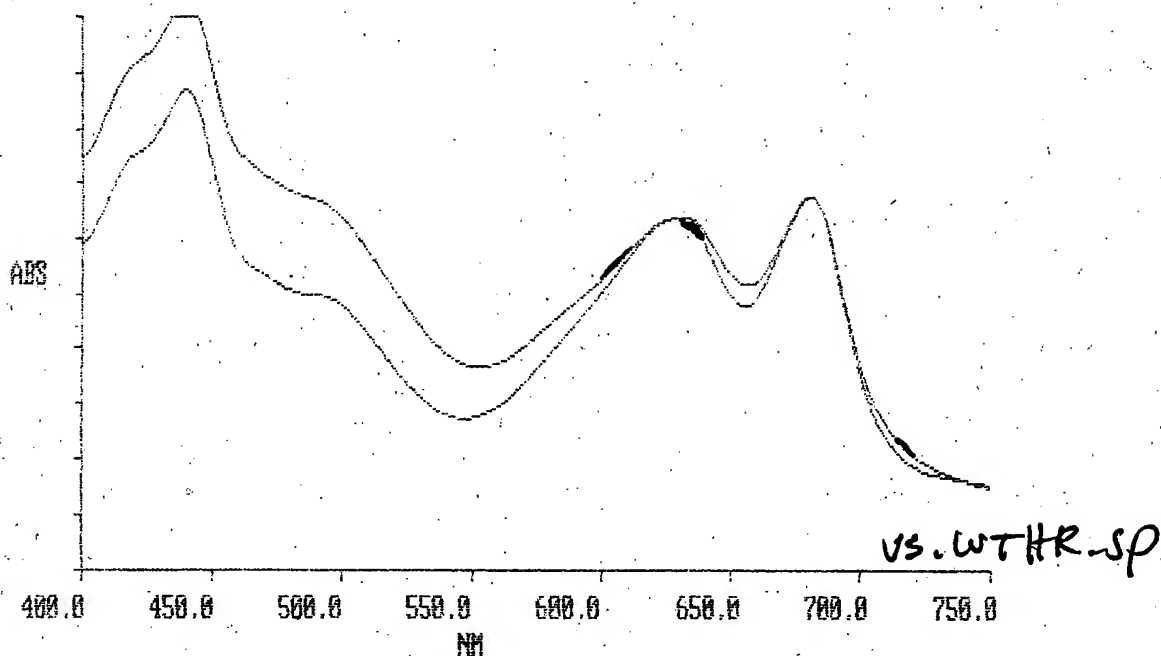
4/30/98. Use stvC-coated beads from Biometra.

(Recombinant stvC may work better).

Experiment repeated as above. got similar but better

results (binding of An323 gave better/stronger fluorescence).

Z: 00323-02; absc 750.0- 400.0; pts 351; int 1.00; ord 0.4615-0.8795; A  
inf: 09:37:04 98/03/23



— *Anabaena PCC7120* (PDS323)

2-L culture in AA@/2 + 3 mM HEPES pH 9.0  
+ Sp20. Grown under HL. bubbled.

JPTG - induced for 3.5 days.  
under HL. bubbled.

An 323

2.273 Ann 3/23/58

by Mary & Steve  
3/30/98

HisTag-StrpTag2 - pLI - CpcA

**Isolation of HisTagged proteins from Anabaena PCC7120**Exhibit IVPage 12 of 15

(This method gets **upper limit** on yield; This is for using cells with wet weight ranging from **1 to 2 grams**. It may be necessary to scale down or up the prep with different amount of cells)

Before starting: reserve the Ti60 angled rotor from Nikaido lab and cool it in the coldroom, and reserve the Ultracentrifuge; make sure that there are as many 23-ml ultra-centrifuge tubes as the number of samples; should have all the buffers ice-cold; should not do more than eight samples at a time.

1. Thaw frozen cell pellet on ice, add ice-cold **buffer 0** to final 18 ml. Vortex to resuspend and homogenize.

2. Add (immediately before French press):

12  $\mu$ l pure  $\beta$ -mercaptoethanol (final 10 mM)

200  $\mu$ l 100x **PMSF stock** (final 1mM)

Invert several times to mix.

3. Transfer cell suspension to the large French Press cell; pass 3x at 18,000 psi ("high" setting dial to 1135); cool on ice afterwards.

4. Transfer cell lysate to a 23-ml ultracentrifuge tube, balance well, spin at 37,000 rpm (130,000 x g) at 4°C for 60 min. in the Ti60 rotor. Promptly take out the tubes after centrifuge.

5. Carefully pour out the supernate to a new 50-ml tube --- take care not to carry over any membrane fraction. (the cell lysate supernate volume should be about 20 ml)

6. Bring up volume to 20 ml with **buffer 0**; spec:

filename: 80330504

50x

7. Use 1 to 2 ml of Ni<sup>2+</sup>-NTA beads suspension to set up column, use of 1.0-cm diam. column is preferred.

8. Carefully pour lysate supernate into column, let settle a bit, then let drip slowly into a new 50-ml tube.

9. Reload flow-thru lysate supernate on the column, let drip slowly.

10. (Resuspend beads in all the following washes):

Wash with 1 column-full (about 20 ml) of **buffer A1**;

Wash with 1 column-full of **buffer B**;

Wash with 1 column-full of **buffer A2**.

Color of the beads after washing:

Beads overloaded?

likely

dark/deep blue

11. Elute HisTagged proteins with 2 ml of eluent **buffer C** (resuspend beads), twice; [elute with another 2 ml of **buffer C** if necessary (do not resuspend beads, and let drip slowly)].

Color of the eluate:

clear deep blue

fairly hard to elute.

6 x 2 ml total.

Beads still quite blue.

13. If the eluate has color, spec from 350 to 750 nm (can not spec with shorter wavelengths because of imidazole).

14. Immediately dialyze samples to eliminate imidazole ---- the 200 mM imidazole in buffer C denatures proteins slightly, and should not be with the proteins for an extended period of time.

Usual dialysis conditions (all should be done at 4°C):  
(all need 50 ml of 2 M Tris HCl pH 8.0, 50 ml of 4 M Na/KCl, and 1 ml of 1 M DTT)

4 liters of 25 mM Tris pH 8.0, 50 mM Na/KCl, 0.5 mM DTT; 0/✓

change buffer 1 or 2 times (depending on number of samples); then

2 liters of 50 mM Tris pH 8.0, 100 mM Na/KCl, 1 mM DTT.

15. Retrieve sample, and spec (use the final dialysis buffer to background).

----- 12.5 ml; some precipitate  
All buffers should be kept at 4°C:

**Buffer 0:**

20 mM Tris HCl pH 8.0  
100 mM Na/KCl

To make 1 liter: Add to 965 ml of MQ-H<sub>2</sub>O  
10 ml of 2 M Tris HCl pH 8.0  
25 ml of 4 M Na/KCl

**Buffer A1:**

20 mM Tris HCl pH 8.0  
100 mM Na/KCl  
20 mM imidazole  
5% glycerol

To make 1 liter: Add to 898 ml of MQ-H<sub>2</sub>O  
10 ml of 2 M Tris HCl pH 8.0  
25 ml of 4 M Na/KCl  
4 ml of 5 M imidazole  
63 ml of 80% glycerol

**Buffer B:**

20 mM Tris HCl pH 8.0  
1 M (!) Na/KCl

To make 1 liter: Add to 740 ml of MQ-H<sub>2</sub>O  
10 ml of 2 M Tris HCl pH 8.0  
250 ml of 4 M Na/KCl

**Buffer A2:**

20 mM Tris HCl pH 8.0  
100 mM Na/KCl  
30 mM imidazole

To make 1 liter: Add to 959 ml of MQ-H<sub>2</sub>O  
10 ml of 2 M Tris HCl pH 8.0  
25 ml of 4 M Na/KCl  
6 ml of 5 M imidazole

**Buffer C:**

20 mM Tris HCl pH 8.0  
100 mM Na/KCl  
200 mM imidazole

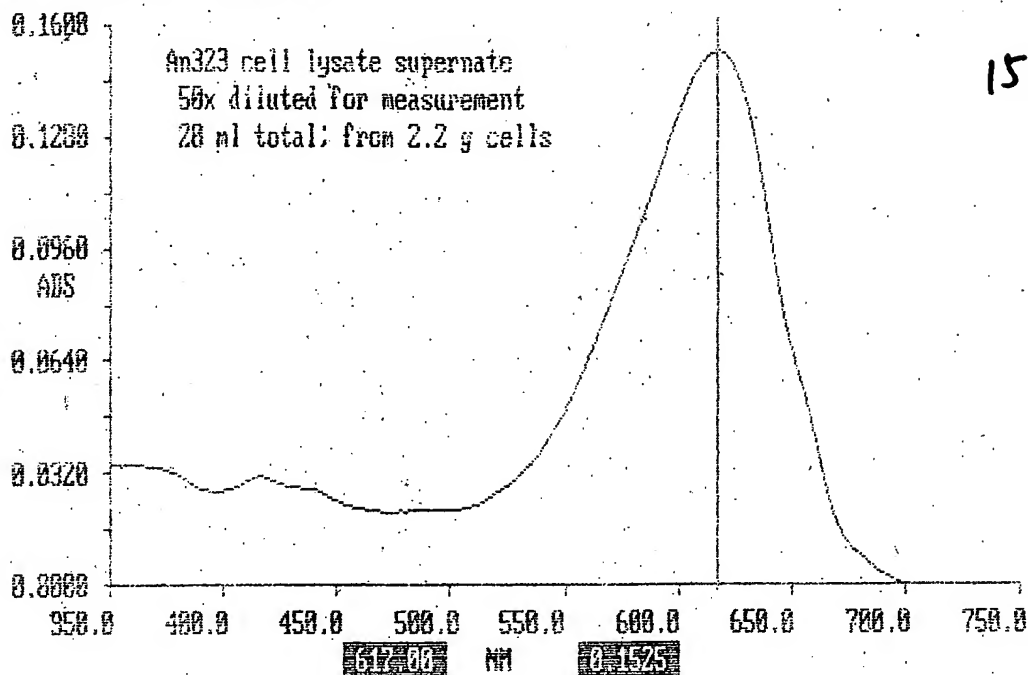
To make 0.5 liter: Add to 462.5 ml of MQ-H<sub>2</sub>O  
5 ml of 2 M Tris HCl pH 8.0  
12.5 ml of 4 M Na/KCl  
20 ml of 5 M imidazole

**Other stock solutions needed:**

100 mM PMSF (Dissolve <sup>0.174g</sup> 1.74 grams of PhenylMethylSulfonyl Fluoride in <sup>10mL</sup> pure EtOH)  
5 M imidazole (keep at 4°C)  
4 M Na/KCl (2 M NaCl + 2 M KCl)  
2 M Tris HCl pH 8.0  
2 M Tris HCl pH 7.0

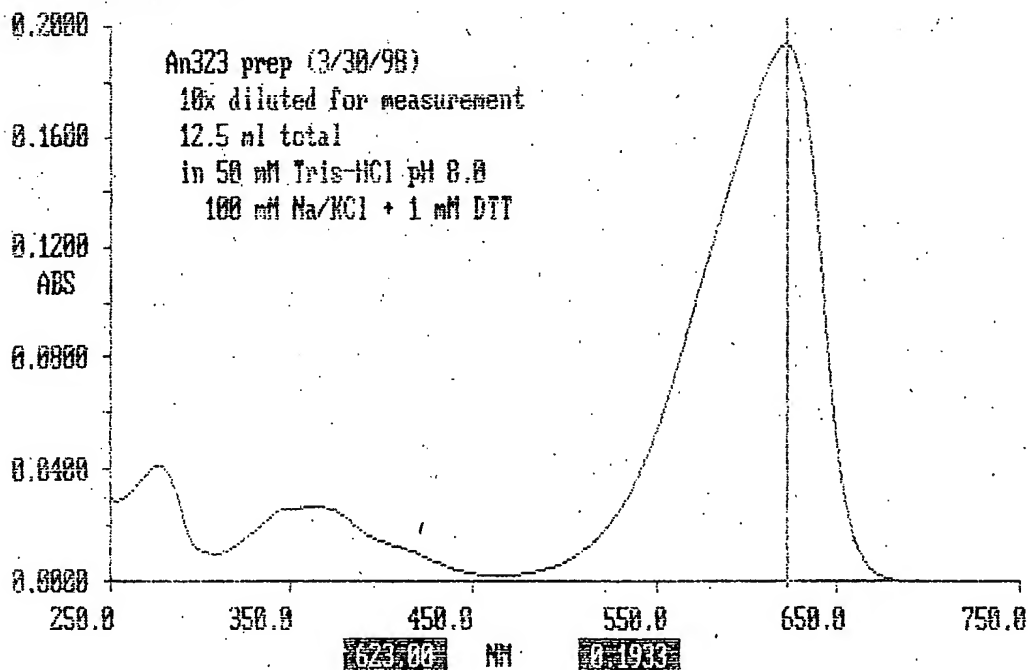


Y: 00330s04; absc 750.0-350.0; pts 481; int 1.00; ord -0.003-0.1525; A  
inf: 14:17:54 98/03/30



Beads overload  
non-elutable pigment. >15.0%

X: 00402s02; absc 750.0-250.0; pts 501; int 1.00; ord -0.000-0.1933; A  
inf: 11:47:00 98/04/02



622 also

1  
11  
SV E323  
VS 7.5  
Am323  
[REDACTED]  
DER  
E323

11  
Am323  
7.5  
E323  
2A  
[REDACTED]